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Combination of Anti-Biofouling and Ion-Interaction by Click Chemistry for Endotoxin Selective Removal from Protein Solution

Jinshan Guo, Fanbo Meng, Xiabin Jing, and Yubin Huang**

Endotoxins (ET, or Lipopolysaccharides (LPS)), as a constituent of the cell wall of gram-negative bacteria, largely exists in biological products, especially recombinant proteins produced by gram-negative bacteria. The removal of ET, even in nanogram quantities, from these biological products before injections is critical due to their potent biological activities causing pyrogeneic and shock reactions in mammals.[1,2] ETs are amphipathic molecules containing anionic and hydrophobic regions, thus their removal often requires cationic ligands immobilized adsorbents. However, proteins (especially acidic proteins) can also be adsorbed by these kinds of adsorbents, leading to the failure of ET selective removal from proteins solutions.[3,4] The traditional way to solve this problem is to use cationic ligands with lower pK_α values, such as poly (ε-lysine) (ε-PL, pK_α = 7.6)[1,2] or its analogues[5] to make the interactions weaker between adsorbents and proteins. However, this method largely limits the species of cationic ligands employed.

It is well known that anti-biofouling materials such as poly(ethylene glycol) (PEG) derivatives[6–8] or zwitterionic polymers[9–12] exhibit perfect protein resistance. Previous research has already shown that after conjugating antibacterial peptides onto oligo(ethylene glycol) (OEG) brushes, the resultant polymer brushes exhibited both antibacterial and antifouling activity.[13] PEGylated poly (ε-lysine) (ε-PL) analogues, containing cationic groups with low pK_α values, also have shown an enhanced capacity for ET selective removal from BSA solution.[14] These successful results inspire us to combine ordinary cationic polymer ligands (high pK_a) with PEG derivatives to reach the high goal of ET selective removal from protein solutions.

In our designation, one of the ordinary polycations, poly (L-lysine) (PLL, pK_a = 9–10), which has stronger positive electricity but less selectivity to the ET-containing protein solution, was chosen as ET-adsorbing part. PEG was also immobilized together with PLL onto the same adsorbent, such as polystyrene microspheres (PS-MS). The anti-biofouling characteristic of PEG is expected to help to reduce the protein adsorption, while the ET adsorbing capacity of PLL could be furthest maintained to realize the ET selective removal from protein solutions.

As the most effective surface/interface reaction,[15–20] click chemistry was applied to immobilize PLL and PEG onto the same carrier conveniently. And 2,2′-bipyridine (BiPy)[21] was employed as chelating agent to remove the residual Cu ions, which is the by-product of copper (I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) and may cause the denaturation of proteins (**Figure 1**a).

To realize click chemistry, a relatively thermal-stable azide-functionalized monomer, 4-azidomethyl styrene (AMS), was

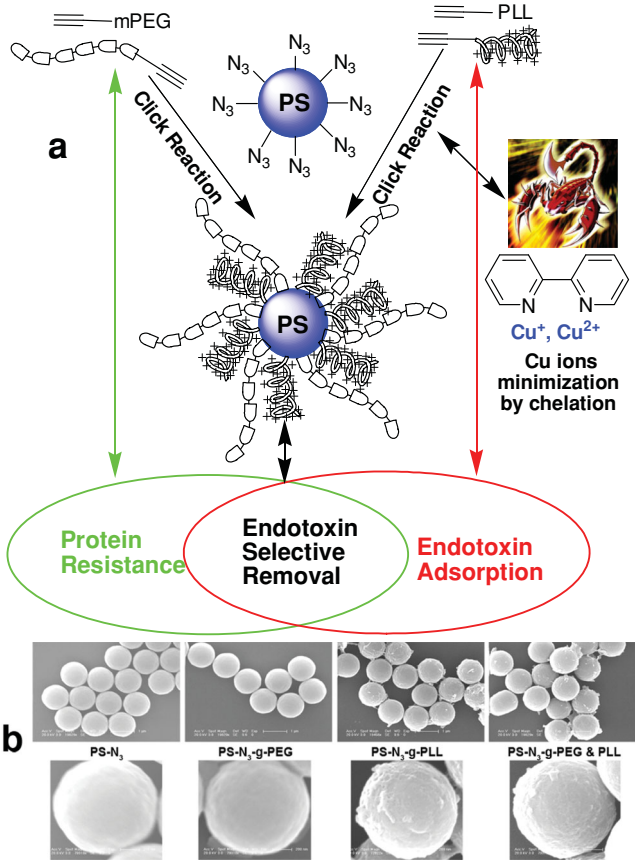

Figure 1. a) Schematic presentation of PLL and PEG immobilized absorbent for endotoxin selective removal from protein solution. b) SEM images of PS-N_3 microspheres (MS) and PS-N_3 MS immobilized with PEG or/and PLL.

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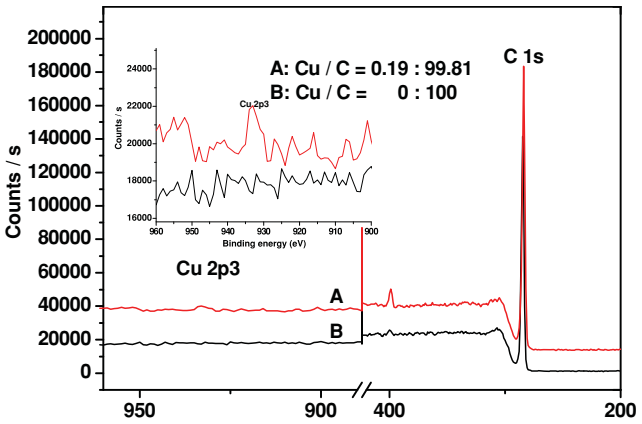

Figure 2. XPS of PPLL30 immobilized PS-N_3 MS using the catalyst system of CuCl (5 eq.)-NaLAC (10 eq.)/Et_3N (32 eq.) (A) and $CuSO_4$ (3 eq.)-NaLAc (10 eq.)/Et_3N (32 eq.) + Bipy (10 eq.) (B).

synthesized (See supporting information, Scheme S1, Figure S1 and S2). The AMS was polymerized together with styrene (S) and divinyl benzene (DVB) by dispersion polymerization at 70 °C to form azide-containing cross-linked polystyrene (PS-N_3) microspheres (MS) (see Scheme S1, Figure S3 and S4 in supporting information). Only PS-N_3 MS with the AMS/S ratio of 1:10 was used as the adsorbent carrier in this paper. The existence and reactivity of azide groups on the surface of PS-N_3 MS was verified by the successful conjugation of alkynyl-rhodamine B (a red colored dye) indicating red fluorescence from fluorescence microscope observation (see Scheme S2 and Figure S5).

Meanwhile, alkyne-terminated PLL (PPLL) (PPLL30, 50, 70, with polymerization degrees [DPs] of 27.6, 49.3, and 70, respectively) and alkyne-terminated PEG (PPEG) (PPEG1000, 2000 and 5000, from mPEGs with molecular weights [MWs] of 1000, 2000, and 5000 Da, respectively) were also synthesized (supporting information). To obtain the optimized condition for surface click reaction, the effect of reaction conditions to the graft efficiency of PPLL on the surface of PS-N_3 MS was investigated by varying the type and amount of catalyst, base or ligand used. The graft efficiencies were indirectly determined by the determination of PPLL concentration in the supernatant by the ninhydrin technique (Table S1 and S2). By overall considering graft efficiency and the Cu ions residual amount in resultant MS, the optimized formulation for these click reactions was determined to be: $CuSO_4$ (3 equiv. to alkyne groups), L-ascorbic acid sodium salt (NaLAc) (10 equiv.), triethylamine (TEA, 32 equiv.) and Bipy (10 equiv.). The reaction was carried out in water at 37 °C for 24 hours under nitrogen atmosphere. Under this condition, the grafting efficiencies were around 50%~70% with nearly undetectable (by XPS, see **Figure 2**) Cu ion residual. We found that addition of base not only reduced the amount of Cu ion residual, but also became a key factor to ensure the development of click reactions in our system (Table S1 and S2). It was reported that the ammonia-ion derived from the amino groups on PPLL could adsorb Cu (I) or Cu (II) ions to form complex. These immobilized Cu (I) ions would no longer catalyze the click reaction.[22] However, by addition of base, the ammonia-ions on PPLL were recovered to free amino groups, and the immobilized Cu (I) ions were then released. The free Cu (I) ions could reach the position of triple bonds and catalyze click reaction between triple bonds and azide groups. It is noted that Bipy here acted not only as base, but also as a kind of chelating agent, which allowed the Cu ions to be removed easily by washing with water (see Figure 1a and Figure 2). Even when more sensitive method, ICP-MS, was used, the detected Cu weight contents in the PS-N_3-g-PLL MS obtained with the participation of Bipy (7.9 ng/mg, B in Figure 2) was also extremely lower than that of the sample obtained without Bipy's participation (352.4 ng/mg, A in Figure 2), further indicating the Cu ions removal effect of Bipy. Although trace amount of Cu ions residual still exists in the samples obtained by the optimized process, its detrimental effect to proteins is very limited, which could be proved by following protein recovery experiments (**Figure 3** and **4**). Previous research about

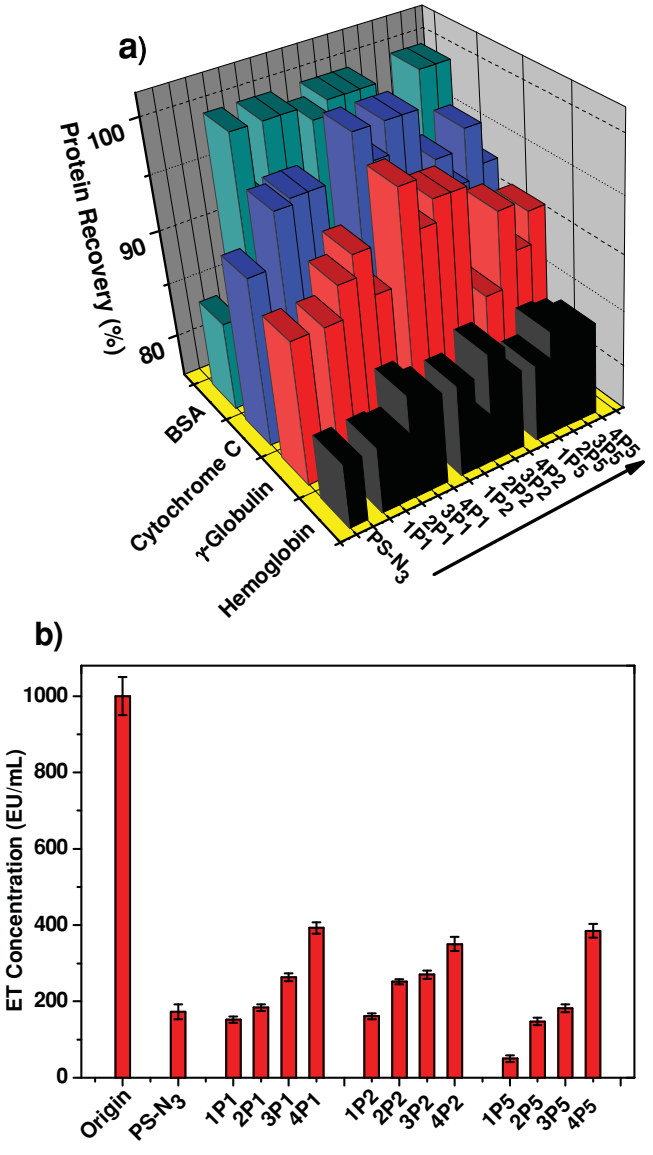

Figure 3. a) Protein recoveries after treatment with different molecular weights of PPEG immobilized PS-N_3 MSs. The concentrations of cytochrome C, γ-globulin, hemoglobin and BSA were 0.278, 0.456, 0.496 and 0.49 mg/mL, respectively; b) ET concentrations after treatment with different molecular weights of PPEG immobilized PS-N_3 MSs.

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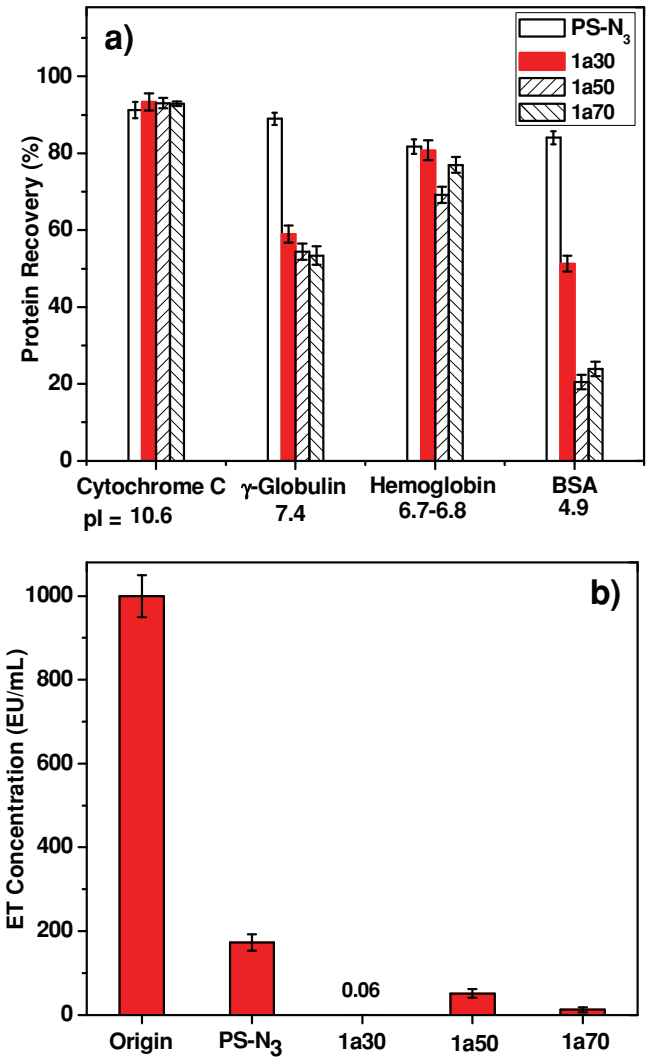

Figure 4. a) Protein recoveries after treatment with different molecular weights of PPLL immobilized PS-N_3 MSs with the same content of amino group. The concentrations of cytochrome C, γ-globulin, hemoglobin and BSA were 0.278, 0.456, 0.496 and 0.49 mg/mL, respectively; b) ET concentrations after treatment with different molecular weights of PPLL immobilized PS-N_3 MSs.

cell-compatible CuAAC, which employed pyridine-neared azide as a copper-chelating azide, provided similar paradigm.[21b]

To confirm whether PLL and PEG can be immobilized onto the surface of PS-N_3 MS at the same time, PPLL and PPEG were labeled with different colored fluorescent dyes. In this work, PPLL was labeled with FITC (green fluorescence), and PPEG was labeled with rhodamine B (red fluorescence) (Scheme S3). After click reaction, both green and red fluorescence could be observed from the resultant MS at exciting wavelengths of 494 and 555 nm, respectively (Figure S6), indicating the successful immobilization of PLL and PEG onto PS-N_3 MS at the same time.

Because ET (lower than 10 KDa) possess smaller molecule weight or size than that of proteins (higher than 10 KDa, always higher than 40 KDa), in the ideal adsorbent model, the chain length of PLL should be shorter than that of PEG, thus the PEG chains could extend to the out-layer of the adsorbent surface and resist proteins which possess big sizes, and at the same time, small-molecular ET molecules could pass through the rift of PEG-riched out-layer and be adsorbed onto the PLL-riched inside layer. In order to thoroughly investigate the modification effect of PLL and PEG on PS-N_3 MS to ET and proteins, the respective ET adsorbing property and protein resistance property of the adsorbent were first studied separately. For this purpose, PEG solely (with different MWs and adding amounts) loaded PS-N_3 MS (PS-N_3-g-PEG) (the samples' names are listed in the Experimental Section below) and PLL solely (with different DPs and adding amounts)loaded PS-N_3 MS (PS-N_3-g-PLL) were also prepared through the same CuAAC reactions (the samples' names are also listed in the Experimental Section below). After immobilization with PEG or PLL, the sizes of resultant MSs remained constant, while the surface coarseness increased, especially in the case of PS-N_3-g-PLL (Figure 1b), further indicating the successful graft of PLL or PEG. The mild variation in surface coarseness of PS-N_3-g-PLL MSs (1a30, 1a50 and 1a70) obtained by using the same weight amount of PPLLs with different DPs (PPLL30, 50 and 70) also can show the PLL graft density decrease trend as 1a30 > 1a50 > 1a70 (Figure S7). The protein recoveries and ET adsorbing capacities of the samples were tested by treating 1 mL of protein or ET solutions with 10 mg of samples.

After immobilizing PEGs with different MWs, the protein recoveries were all higher than that of PS-N_3 MS itself. In the cases of BSA and cytochrome C solutions, some of the protein recoveries could even reach to 100% (Figure 3a). The increase of protein recoveries had a tendency of PEG2000 > PEG5000 > PEG1000. Although the protein recoveries are not always positively correlated with the amount of PEG loaded, the protein recoveries of MSs with PPEG adding amounts of 100, 150 and 200 mg/g (2P1, 3P1, 4P1; 2P2, 3P2, 4P2; 2P5, 3P5, 4P5) are all higher than that of PPEG adding amount of 50 mg/g (1P1, 1P2, 1P5), for small amount of PEG grafted or low PEG graft density can not reach sufficient surface coverage of PEG on the adsorbent to meet effective protein resistance. Even after PEGylation, the protein recoveries of hemoglobin were still not very high, which maybe caused by the natural denaturation of methemoglobin (the state of hemoglobin received) in the air. As shown in Figure 3b, after immobilizing with low amounts of PEG, the ET adsorption capacities were even higher than that of PS-N_3 MS itself. However, the ET adsorption capacities decreased sharply with increasing PEG amounts, showing the significant shielding effect of PEG to ET, too. It is implied that an optimized PEG loading amount is required to maintain both protein recovery and ET adsorption capacity at the suitable level. From the above results, PPEG adding amount of 50 mg/g was excluded in further studies, and PPEG 2000 and 5000 were determined to show better comprehensive performance and were chosen as the protein-resistant part in further studies in combination with PLL.

For the convenience of comparison between PPLLs with three different DPs (PPLL30, 50 and 70), and to reduce the workload of research, the added amount of PPLL to PS-N_3 MS was fixed at 1.0 mmol NH_2/g, and the amino contents of PS-N_3-g-PLL MSs were all around 0.5 mmol NH_2/g. After immobilizing with

PLL with different DPs, the protein recoveries of γ-globulin, hemoglobin, and BSA decreased. Especially in the cases of γ-globulin and BSA, the protein recoveries decreased sharply from nearly 85% to lower than 60%, some of them even to lower than 30%, which were much lower than that of PS-N_3 MS itself (Figure 4a). Since PLL has a pK_α of 9-10, an intense interaction should exist between PLL and the acidic proteins, resulting in vast decrease of protein recovery. In the case of cytochrome C (with high pI value as 10.6, which is higher than the pK_α value of PLL), PS-N_3-g-PLL MS even exhibited repulsion to cytochrome C, resulting in an increase of protein recovery. Among PPLLs with three different DPs, PPLL30 exhibited highest protein recovery (Figure 4a). With the amino contents of PS-N_3-g-PLL MSs kept nearly the same, although shorter chain length implies higher graft density, only the amino groups in the front layer of the surface have big chance to interact with big-sized proteins. While in the case of samples with longer-chain PLL, proteins have more chance to pass through the front layer and interact with the inside amino groups, causing the decrease of protein recoveries. After treatment with PS-N_3-g-PLL MSs, the ET concentrations sharply decreased to lower than 100 EU/mL from original 1000 EU/mL, with a trend of 1a30 > 1a50 >1a70, for shorter chain length implies higher graft density. The one treated with PPLL30 loaded PS-N_3 MS (1a30) even reduced ET concentration to nearly zero, indicating the intense ionic interaction between PLL and ET. PS-N_3 MS itself also exhibited some ET adsorbing capacity for its hydrophobicity (Figure 4b). Among the three PPLLs, PPLL30 exhibited the highest ET adsorbing capacity and was chosen to combine with PEG for the further study.

Furthermore, we have also immobilized PPEG 2000 or 5000 and PPLL30 together onto PS-N_3 MSs (PS-N3-g-PEG & PLL, the samples' names and their amino contents are listed in the Experimental Section below), hoping the combined effect would bring sufficient ET adsorbing capacity and a suitable protein recovery to the adsorbent. Again, the two properties of the adsorbent were first studied separately. The results showed that the existence of PLL in PS-N_3-g-PEG & PLL MSs would increase the protein adsorption. However, with the help of PEG, the strong interaction between PLL and proteins could be well controlled by PEG shielding, providing the acceptable protein recovery (**Figure 5**a). On the other hand, when ET-alone solution was treated with PS-N_3-g-PEG & PLL MSs, most of the adsorbents showed similar and efficient ET adsorption capacities as that of the PS-N_3-g-PLL MSs. The existence of PEG, especially PEG 2000, did not show significant effect on ET adsorption capacity in most of the cases (Figure 5b). It has to be noted that the ionic interaction between adsorbents and ETs would be overshadowed and leading to the decrease of ET adsorption capacity, when large amount of PEG 5000 was applied, for large amount of long PEG chains will tangle together and prevent ET molecules pass through PEG-riched out layer and be adsorbed onto the PLL-riched inside layer. These separate studies indicate that the proper combination of PLL and PEG could well display the ET adsorbing property of PLL and protein resistance property of PEG together without showing the disadvantages when PLL and PEG were used alone.

For the practical examination, the ET selective removal property of the final obtained PS-N_3-g-PEG & PLL MSs was

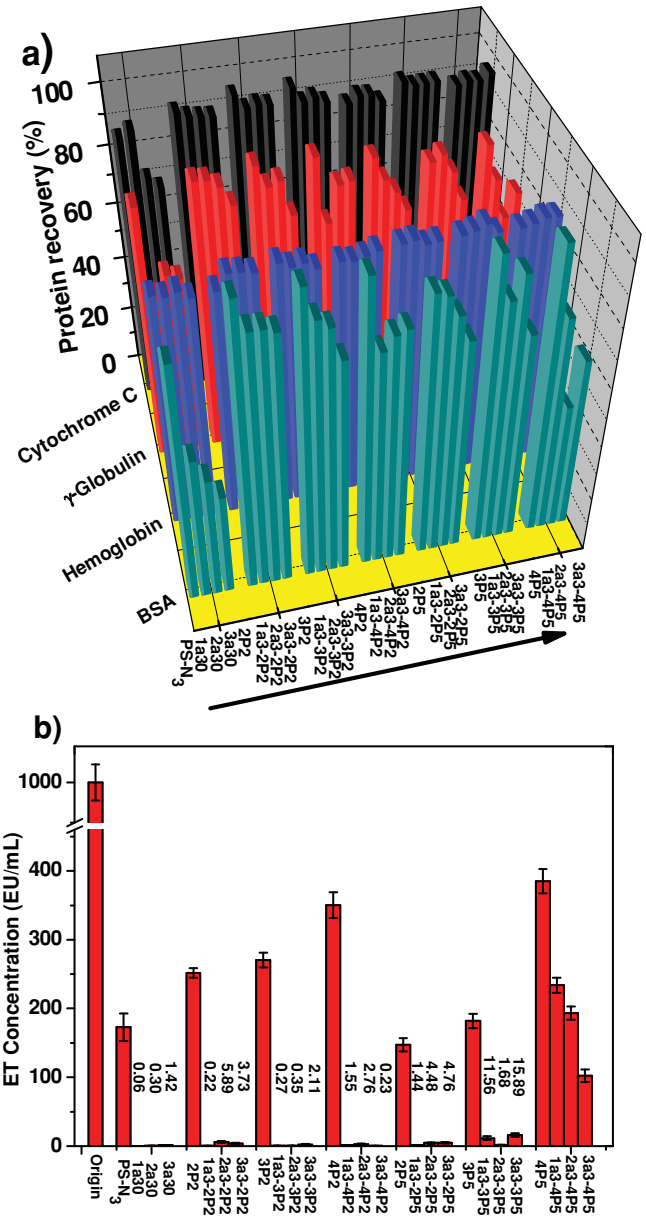

Figure 5. a) Protein recoveries after treatment with PPLL and PPEG immobilized PS-N_3 MSs; b) ET concentrations after treatment with PPLL and PPEG immobilized PS-N_3 MSs.

investigated using ET mixed BSA solution as an example. As shown in **Figure 6**a, after treating with 10 mg PS-N_3-g-PEG and PLL MSs, the ET concentrations remained in the ET mixed BSA solution (1 mL) decreased vastly from 14000 EU/mL to lower than 5000 EU/mL, exhibiting ET adsorbing capacities higher than 9×10^5 EU/g adsorbent. At the same time, PS-N_3-g-PEG & PLL MSs showed proper BSA recovery, displaying the expected ET selective removal property from protein solution. Although PS-N_3-g-PLL MSs have the best ET adsorption capacity, the BSA recovery of this adsorbent was unsatisfactory. Furthermore, perfect ET adsorbing effect could be obtained by increasing the amount of PS-N_3-g-PEG & PLL MSs used. The results showed

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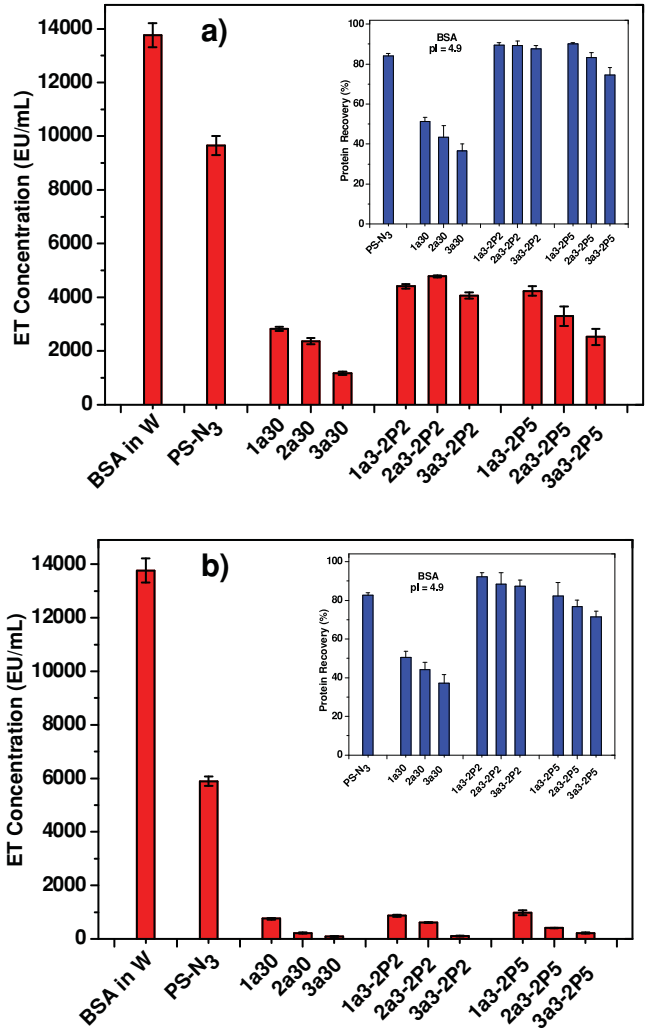

Figure 6. a) ET adsorbing and BSA recovery results by treating 1 mL of ET-containing BSA solution with 10 mg of adsorbents; b) ET-adsorbing and BSA recovery results by treating 1 mL of ET-containing BSA solution with 20 mg of adsorbents.

that when the adsorbent amount of PS-N_3-g-PEG & PLL MSs was increased from 10 mg to 20 mg in the same ET mixed BSA solution, the concentration of remained ET could sharply decrease to less than 100 EU/mL, while the BSA recoveries kept nearly unchanging at high level (Figure 6b). It would be practically useful for the complete removal of ET (like less than 0.5 EU/mL) without significant protein loss.

In conclusion, the successful combination of anti-biofouling and ion-interaction for endotoxin ET selective removal from protein solution was realized via the immobilization of two ordinary polymers, PEG and PLL, together onto azide-functionalized polystyrene microspheres (PS-N_3 MS) using surface click reaction. The toxic copper ions residual was successfully minimized by the addition of 2,2′-bipyridine (BiPy). By adjusting the molecular weights and adding various amounts of PLL and PEG, the optimized PS-N_3-g-PEG & PLL MSs exhibited perfect ET selective removal property from protein solutions, even in the case of acidic protein like BSA. Before this, only the polycations with low pK_α values that have weak interaction with proteins could be used in ET selective removal from protein solutions. Our delightful findings break the old patterns, largely expand the series of polycations that can be employed in the application of ET selective removal, and this also could be extended to the application fields of ET selective removal from other bioactive molecules solutions, such as DNA, RNA, etc.

Experimental Section

PS-N_3-g-PEG samples: PPEGs with three different MWs of 1000, 2000 and 5000 Da were abbreviated as P1, P2 and P5. For each of them, four different amounts (50, 100, 150 or 200 mg/g) were used for the immobilization, the resultant PS-N3-g-PEG MSs were named as **1P1**, **2P1**, **3P1**, **4P1**; **1P2**, **2P2**, **3P2**, **4P2**; **1P5**, **2P5**, **3P5**, **4P5** correspondingly, here the first number (1, 2, 3, 4) represents the amount of PPEG used, 50, 200, 150, and 200 mg/g, respectively.

PS-N_3-g-PLL samples: PPLL 30, 50 and 70 were abbreviated as a30, a50 and a70. For each of them, three different amounts (1.0, 1.5 and 2.0 mmol NH_2/g) were used for the immobilization, the resultant PS-N3-g-PLL MSs were named as **1a30**, **2a30**, **3a30**; **1a50**, **2a50**, **3a50**; **1a70**, **2a70** and **3a70** correspondingly, the amino contents of them are 0.59, 0.88, 1.42; 0.62, 1.07, 1.49; 0.50, 0.70, and 0.91, respectively, here the first number (1, 2, 3) represents the amount of PPLL used, 1.0, 1.5, and 2.0 mmol NH_2/g, respectively.

PS-N_3-g-PEG & PLL samples: PS-N3-g-PEG & PLL samples were obtained by immobilizing different amounts of PPLL30 (a30) (1.0, 1.5 or 2.0 mmol NH2/g) and PPEG2000 (P2) or 5000 (P5) (100, 150 or 200 mg/g) onto PS-N3 MS at the same time, the name of the sample was based on the MWs and amounts of PEG and PLL used, the amino groups contents (mmol NH_2/g) of every samples are also given in the brackets just after the corresponding names of the samples, as shown below: **1a3-2P2** (0.68, here 1a3 reapects to 1a30 as descriped before), **1a3-3P2** (0.51), **1a3-4P2** (0.51), **2a3-2P2** (0.92), **2a3-3P2** (0.88), **2a3-4P2** (0.95), **3a3-2P2** (1.19), **3a3-3P2** (1.20), **3a3-4P2** (1.24); **1a3-2P5** (0.52), **1a3-3P5** (0.52), **1a3-4P5** (0.54), **2a3-2P5** (0.82), **2a3-3P5** (0.79), **2a3-4P5** (0.91), **3a3-2P5** (1.14), **3a3-3P5** (1.17), **3a3-4P5** (1.21).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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